

B/O Form PTO-1390 Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a Filing Under 35 USC 371		Attorney's Docket Number HOGL3001/REF
International Application Number PCT/SE00/01767	International Filing Date September 13, 2000	U.S. Application Number (if known) 10/070412
<i>Title of Invention</i> DNA CONSTRUCT AND ITS USE		
<i>Applicant(s) for DO/EO/US</i> HOGlund et al.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items under 35 USC 371:		
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 USC 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 USC 371. 3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed 35 USC 371(c)(2). <ol style="list-style-type: none"> a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 USC 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 USC 371(c)(4)). (<input checked="" type="checkbox"/> Executed <input type="checkbox"/> Unexecuted). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)). <p>Items 11 to 16 below concern other document(s) or information included:</p> <ol style="list-style-type: none"> 11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English translation of the international application under 35 U.S.C. 154(d)(4) 20. <input checked="" type="checkbox"/> Other items or information: Sequence Listing and Application Data Sheet 		

Application Number (if Known) 10/070412	International Application Number PCT/SE00/01767	Attorney's Docket Number HOGL3001/REF	
		Calculations	
<p>1. The following fees are submitted:</p> <p>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</p> <p><input checked="" type="checkbox"/> Neither International Preliminary Examination Fee (37 CFR 1.482) nor International Search Fee (37 CFR 1.445(a)(2)) paid to USPTO \$1040.00</p> <p><input type="checkbox"/> Search report has been prepared by the EPO or JPO \$890.00</p> <p><input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) \$710.00</p> <p><input type="checkbox"/> No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but International Search Fee paid to USPTO (37 CFR 1.445(a)(2)) \$740.00</p> <p><input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00</p>			
ENTER APPROPRIATE BASIC FEE AMOUNT		\$ 1,040.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).			
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total Claims	20	-20 =	0
Independent Claims	1	-3 =	0
Multiple Dependent Claims (if applicable)		+ \$280.00	
TOTAL OF ABOVE CALCULATIONS			
Reduction by ½ for filing by small entity, if applicable. Small Entity Status is asserted pursuant to 37 CFR 1.27 for this application.		\$ 520.00	
SUBTOTAL \$ 520.00			
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			
TOTAL NATIONAL FEE			
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property.		\$ 40.00	
TOTAL FEES ENCLOSED		\$ 560.00	
		Amount to be:	Refunded: Charged:

- a. A check in the amount of \$560.00 to cover the fees is enclosed.
- b. Please charge my **Deposit Account Number 02-0200** in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to **Deposit Account Number 02-0200**. A duplicate copy of this sheet is enclosed.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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23364
PATENT TRADEMARK OFFICE

DATE: March 15, 2002

Respectfully submitted,



Attorney for Applicant: Richard E. Fichter
Registration Number: 26,382



4

STATEMENT

I, Brita Nilsson, hereby certify that the information recorded in computer readable form is identical to the written sequence listing as filed with the original PCT application PCT/SE00/01767.

Signed

A handwritten signature in black ink, appearing to read "Brita Nilsson".

Brita Nilsson

April 10, 2002

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of : Attention: PCT OFFICE
HOGLUND et al. :
U.S. National Phase of PCT/SE00/01767 :
Entry papers filed herewith March 15, 2002 :
For: DNA CONSTRUCT AND ITS USE :

**PRELIMINARY AMENDMENT
AND INFORMATION DISCLOSURE STATEMENT**

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The present application is the U.S. national phase of international application number PCT/SE00/01767. The following amendments pertain to the claims as amended.

Please note that the amended page 1 (new claim set) attached to the International Preliminary Examination Report (Annexes) and submitted herewith, have replaced the originally filed page 8 of the application. The claims to be examined and amended by this preliminary amendment are found on amended page 1 (of the new claim set).

Please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please add the attached ABSTRACT OF THE DISCLOSURE to the application.

IN THE CLAIMS:

Please replace claims 3 and 5-6 with the following amended claims.

3(Amended). Transgenic oilseed plant cell according to claim 1, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and acyl transferase activity.

5(Amended). Transgenic oilseed plant cell according to claim 1, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

6(Amended). Transgenic oilseed plant cell according to claim 1, wherein the cell expresses xanthophyll.

Please add the following new claims to the application.

10(New). Transgenic oilseed plant cell according to claim 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and acyl transferase activity.

U.S. National Phase of PCT/SE00/01767

11(New). Transgenic oilseed plant cell according to claim 2, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

12(New). Transgenic oilseed plant cell according to claim 3, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

13(New). Transgenic oilseed plant cell according to claim 4, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

14(New). Transgenic oilseed plant cell according to claim 10, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

15(New). Transgenic oilseed plant cell according to claim 2, wherein the cell expresses xanthophylls.

16(New). Transgenic oilseed plant cell according to claim 3, wherein the cell expresses xanthophylls.

17(New). Transgenic oilseed plant cell according to claim 4, wherein the cell expresses xanthophylls.

18(New). Transgenic oilseed plant cell according to claim 5, wherein the cell expresses xanthophylls.

19(New). Transgenic oilseed plant cell according to claim 10, wherein the cell expresses xanthophylls.

U.S. National Phase of PCT/SE00/01767

20(New). Transgenic oilseed plant cell according to claim 14, wherein the cell
expresses xanthophylls.

REMARKS

Applicants have amended the claims in order to reduce the initial filing fee by deleting the multiple dependent claims from the application. Some of this subject matter has been reintroduced as dependent claims 10-20. Applicants retain the right to reintroduce any subject matter canceled by the present Amendment at any time during the prosecution of this application or any further application claiming benefit of this application.

Applicants have amended the application to substitute the originally filed page 8 with the amended claim set page 1 attached to the International Preliminary Examiner Report (Annexes) and included in the application as filed herewith. Also, an Abstract of the Disclosure has been added to the application.

Applicants are submitting herewith a copy of the International Search Report which issued on International Application No. PCT/SE00/01767, of which the present application is the U.S. national phase which was published in English. All of the publications cited in the International Search Report are listed on the attached Form PTO-1449. It is Applicants' understanding that, under the procedures of the PCT, copies of the cited publications will have been supplied to the U.S. Patent Office by the International Bureau. However, the Examiner is invited to contact the undersigned attorney if additional copies are necessary or would facilitate examination of the present application.

Otherwise, the Examiner is respectfully requested to return an initialed and dated copy of the attached Form PTO-1449 to confirm that all publications listed thereon have been considered and made officially of record in the file of this application.

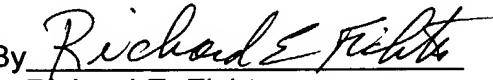
Applicants understand that, under the procedures of the PCT, a copy of the priority document (SE 9903336-7, filed 17 September 1999) will have been supplied to

U.S. National Phase of PCT/SE00/01767

the U.S. Patent Office pursuant to Rule 17 of the PCT Regulations. It is therefore respectfully requested that the first Official Action in the present application contain an indication that the appropriate priority document is in the file of this application.

In view of the above amendments, an early action on the application is now in order and is most respectfully requested.

Respectfully submitted,
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REF:kdd
PA01.wpd

DATE: March 15, 2002

Marked-Up Version Showing Changes Made

IN THE CLAIMS:

Please replace claims 3 and 5-6 with the following amended claims.

3(Amended). Transgenic oilseed plant cell according to claim 1 [or 2], wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, and acyl transferase activity.

5(Amended). Transgenic oilseed plant cell according to [any one of claims 1 - 5] claim 1, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

6(Amended). Transgenic oilseed plant cell according to [any one of claims 1 - 5] claim 1, wherein the cell expresses xanthophyll.

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Abstract

A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a 5 nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme 10 activity is preferably a peptide with b-carotene C-4-oxygenase activity, e.g. from the alga *Haematococcus pluvialis*.

Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin; transgenic oilseed plant-produced xanthophyll; transgenic oilseed plant-produced canthaxanthin; transgenic oilseed plant-produced 15 astaxanthin; and transgenic oilseed plant-produced astaxanthin esters.

3/Pr/S

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DNA construct and its use.

The present invention relates to a new DNA construct for transformation into oilseed plants. The DNA construct comprises nucleotide sequences encoding peptides with enzyme activities necessary for the high-level production and esterification of keto group-containing xanthophylls in oilseed plants.

Background of the invention

Carotenoids are produced *de novo* by plants, fungi, algae and some bacteria. A number of biosynthetic steps are needed for the biological production of the carotenoids. There are two chemically different groups of carotenoids, namely carotenes containing only carbon and hydrogen molecules and xanthophylls containing oxygen in the molecule in addition to carbon and hydrogen.

The xanthophylls, and particularly astaxanthin (3,3'-dihydroxy- β - β -carotene-4,4'-dione), are often colored pigments and are used as such or as anti-oxidants.

Carotenes are biological precursors for the production of the oxygen-containing xanthophylls. There are two types of enzymes responsible for the introduction of hydroxy groups and keto groups into the carotenes, namely hydroxylases and ketolases, respectively.

The keto group-containing xanthophyll astaxanthin, which has keto and hydroxy groups, is biosynthetically produced from beta-carotene.

Large-scale production of xanthophylls from natural sources is at present performed by AstaCarotene AB, Gustavsberg, Sweden, by cultivation of the alga *Haematococcus pluvialis* for the production of astaxanthin in esterified form.

It would be desirable to be able to produce keto group-containing xanthophylls particularly astaxanthin, in oilseed plants. Oilseed plants have naturally β -carotene hydroxylases but lack β -carotene C-4-oxygenase enzymes or ketolases.

Description of the invention

The present invention provides DNA constructs enabling and promoting production of keto group containing xanthophylls, especially astaxanthin, in oilseed plants, such as rape, sunflower, soybean and mustard. The DNA construct is transformed into the oilseed plant cell for expression of a protein or fused protein which has an enzyme activity enabling keto group insertion into a carotene or hydroxy carotene for the biosynthetic production of a keto group containing xanthophyll, such as cantaxanthin (β , β -carotene-4,4'-dione) and/or astaxanthin. Use is thus made of the biosynthetic pathway of the oilseed plant to

produce carotenoids. The naturally occurring synthesis of carotenoids involves a number of enzymes, namely 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-5 cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase. Genes coding for peptides having these enzymatic activities may be inserted into the DNA construct of the invention, one or several per construct, to promote high-level production in the transgenic oilseed plant. In case only one enzyme coding gene is inserted per plant, two or more plants may be sexually interbred to produce plants containing all the desired enzyme activities.

10 Thus, the present invention is directed to a DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

15 In a preferred embodiment of the invention the DNA construct additionally comprises between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

20 The DNA construct is preferably such that the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production is selected from the group consisting of peptides with 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase activity. To 25 promote esterification of astaxanthin a nucleotide sequence coding for a peptide with acyl transferase activity may be included in the group.

30 In a preferred embodiment of the DNA construct according to the invention the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β -carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

An example of the DNA construct of the invention is presented in the sequence listing as SEQ ID NO:1 and in Fig.1.

The present invention is also directed to a transgenic oilseed plant cell comprising the DNA construct of the invention, and preferably the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

The invention is additionally directed to transgenic oilseed plant-produced 5 xanthophyll, e.g. canthaxanthin and astaxanthin.

A preferred aspect of the invention is directed to transgenic oilseed plant-produced astaxanthin esters.

The present invention will now be illustrated with reference to the DNA construct disclosed in the sequence listing and in Fig.1, and the following description of 10 embodiments. However, the invention is not limited to these exemplifications.

Short description of the drawings

Fig.1 illustrates the nucleotide sequence of the DNA construct comprising the napin promoter, the chloroplast localization signal, the N-terminally truncated β -carotene C-4-oxygenase gene and the termination sequence, and the deduced amino acid sequences of the transit peptide 15 and the β -carotene C-4-oxygenase.

Description of embodiments

The invention is illustrated by production of astaxanthin in the seed of oilseed rape. The astaxanthin produced in the seed of the transgenic plant is extracted as part of the 20 extracted oil. By use of conventionally used protocols for *Agrobacterium tumefaciens* mediated transformation such as described by (Hoekema et al. 1983, An et al. 1986, Fry et al. 1987, DeBlock et al. 1988, Radke et al. 1988, or Moloney et al. 1989) transgenic plants are 25 produced having a chimeric DNA construct that is genetically inherited and is able to produce astaxanthin. The nucleotide sequence of the chimeric DNA construct consist of four parts of different genetic origin namely: (1) a promoter, (2) a localization signal, (3) a β -carotene C-4-oxygenase coding region and (4) a termination sequence.

The napin promoter directs transcription to the seed of oilseed rape (Stålberg et al 1996). This promoter was coupled to a localization signal similar but not identical to a 30 transit peptide (TP) of RbcS1a (Krebbers, 1988) that directs the translated product of a fused gene to the chloroplast. The promoter and the TP sequence were ligated to a part of the coding sequence of a ketolase gene BCK (Kajiwara et al. 1995). This enzyme oxygenates β -carotene to canthaxanthin, (Fraser et al. 1997). The chimeric DNA construct was then coupled to a suitable termination sequence, e.g. that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end)(Bevan et al. 1983), as illustrated in Fig.1.

Cellular storage of Astaxanthin

The storage of large amounts of free astaxanthin in plants will be difficult due to toxic effects of the molecule as it intercalates in the plant membranes. An effective esterification of astaxanthin to fatty acids enables storage of the esterified molecules in triacylglycerol containing oleosomes. Thus, an acyl transferase can be claimed to be of fundamental importance for the process, as is proteins that can mediate transport of different forms of astaxanthin from the chloroplast to the vesicles.

Sequences and oligonucleotides used in the construction of the DNA construct

1. *Napin promoter* (GeneBank ACCESSION No. J02798)

This promoter sequence, a 1145 base pair fragment including the 5' leader sequence has a unique HindIII site at the 5' end. The 3' end was synthesized with an additionally 6 nucleotide BamHI site.

2. *Transit peptide similar to RBCS1a* (GeneBank ACCESSION No. X13611, X14565)

The transit peptide (TP) was amplified by PCR from -28 to the end of the transit cleavage aa=54/55 site of the RbcS1a gene. The 5' end was synthesized with a BamHI site and similarly the 3' sequence was synthesized with a XbaI site. The two following oligonucleotides were used for the PCR amplification.

BamHI

20 5' primer: TP1 5'AGAC **GGATCC** TCAGTCACACAAAGAGTA 3'

SacI XbaI

3' primer: TP2 5'GTTC **GAGCTC** TCTAGA CATGCAGTTAACGC 3'

3. *BCK (β -carotene C-4 oxygenase)* (Genebank ACCESSION No. D45881)

25 The BCK fragment was amplified by PCR including a 5' XbaI site and was ligated to the TP already described. The 5' primer (BCK1) used for PCR, is homologous to the BCK sequence from nucleotide 264 and the 3' oligonucleotide (Ax40) ends with a stop codon and was synthesized with a SacI restriction site for cloning. The synthesized fragment was fused to the TP as shown in Fig 1.

30 Oligonucleotides used for PCR:

XbaI

5' primer: BCK1 5'ACAG **TCTAGA** ATGCCATCCGAGTCGTCA 3'

SacI

3' primer: AX40 5'CACCGAGCTCCATGACACTCTTGTGCAGA 3'

Description of SEQ ID NO:1 and SEQ ID NO:2

The sequences shown in Fig. 1 are the same as the two sequences which are shown in the sequence listing.

The SEQ ID NO:1 is a nucleotide sequence composed of the following features:

5

Nucleotide No.

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15

Cloning site HindIII	1-6
Napin Promoter	1-1145
Cloning site BamHI	1146-1151
Transit peptide leader	1152-1178
Transit peptide coding	1179-1347
Cloning site XbaI	1348-1353
β -carotene C-4-oxygenase	1354-2217
β -carotene C-4-oxygenase 3' untranslated	2218-2266
Cloning site SacI	2267-2272
Nopaline synthetase termination	2273-2536
Cloning site EcoRI	2538-2543

The SEQ ID NO: 2 is a deduced amino acid sequence of the fusion protein of the transit peptide and the peptide with β -carotene C-4-oxygenase activity.

References

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5 Arabidopsis-thaliana using a binary vector system. Plant Physiology 81 (1) 301-305.

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transgenic plants Plant Physiology 91:2, 694-701.

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15 enzymes. J Biol Chem. Mar 7;272(10):6128-35.

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20 Hoekema A, Hirsch PR, Hooykas PJJ Schilperoort, (1983). A binary vector strategy based on
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25 1.7 S storage protein, napin, from Brassica napus. J. Biol. Chem. 262 (25), 12196-12201.

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5 Moloney M, Walker JM and Sharma KK, (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Reports 8:238-242.

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10 Pua E-C, Mehra-Palta A, Nagy F and Chua N-H, (1987). Transgenic plants of *Brassica napus*. Biotechnology vol 5, 815-817.

15 Stålberg K, Ellerstöm M, Ezcurra I, Ablov S, Rask L (1996). Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. Planta 199(4):515-9.

8

Claims

1. Transgenic oilseed plant cell containing a DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of the oilseed plant, a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant, a 5'-truncated beta-carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis* and a transcriptional termination region.
2. Transgenic oilseed plant cell according to claim 1, wherein the cell additionally contains at least one DNA construct selected from DNA constructs comprising in 10 the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of the oilseed plant, a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in the oilseed plant and a transcriptional termination 15 region.
3. Transgenic oilseed plant cell according to claim 1 or 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β-carotene hydroxylase, and acyl transferase activity.
4. Transgenic oilseed plant cell according to claim 1, wherein the nucleotide sequence of the DNA construct is SEQ ID NO:1.
- 25 5. Transgenic oilseed plant cell according to any one of claims 1 - 5, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.
6. Transgenic oilseed plant cell according to any one of claims 1 – 5, wherein the cell expresses xanthophylls.
7. Transgenic oilseed plant cell according to claim 6, wherein a xanthophyll is 30 canthaxanthin.
8. Transgenic oilseed plant cell according to claim 6, wherein a xanthophyll is astaxanthin.
9. Transgenic oilseed plant cell according to claim 8, wherein the astaxanthin comprises astaxanthin esters.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

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Published:

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WO 01/20011 A1

(54) Title: DNA CONSTRUCT AND ITS USE

(57) Abstract: A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme activity is preferably a peptide with β-carotene C-4-oxygenase activity, e.g. from the alga *<u>aematococcus pluvialis</u>*. Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin, and a transgenic oilseed plant-produced xanthophyll, such as canthaxanthin or astaxanthin, and also astaxanthin esters.

10/070412

WO 01/20011

PCT/SE00/01767

1/3

Napin promoter

AAGCTTCTTCATCGGTGATTGATTCCCTAAAGACTTATGTTCTTATCTTGCTTCTGA
GGCAAGTATTCAAGTACCAAGTTACCACTTATATTCTGGACTTCTGACTGCATCCTCATT
TTTCCAACATTTAAATTCACTATTGGCTGAATGCTTCTTGTGAGGAAGAACAAATT
CAGATGGCAGAAATGTATCAACCAATGCATATATAACAAATGTACCTCTTGTCTCAAAAC
ATCTATCGGATGGTCCATTGCTTGTCAATTGACTACTTATATTATTAC
TCCTCTTATTACTATTTCATGCGAGGTTGCCATGTACATTATTTGTAAGGATTGAC
GCTATTGAGCGTTTTCTCAATTCTTATTAGACATGGGTATGAAATGTGTGTTA
GAGTTGGGTTGAATGAGATATACTCAAGTGAAGTGGCATACCCTCTCGAGTAAGGAT
GACCTACCCATTCTGAGACAAATGTTACATTAGTATCAGAGTAAATGTGTACCTAT
AACTCAAATTGATTGACATGTATCCATTCAACATAAAATTAAACCAGCCTGCACCTGCA
TCCACATTCAAGTATTTCAAACCGTTGGCTCCTATCCACCGGGTGTAAAGACGGA
TTCCGAATTGGAAGATTGACTCAAATTCCAATTATATTGACCGTGACTAAATCAA
CTTTAACTCTATAATTCTGATTAAGCTCCAATTATATTCCAACGGCACTACCTCCA
AAATTATAGACTCTCATCCCTTTAAACCAACTAGTAAACGTTTTTTTTAATT
TATGAAGTTAAGTTTACCTTGTAAAAAGAATCGTCATAAGATGCCATGCCAGA
ACATTAGCTACACGTTACACATAGCATGCAGCCGGAGAATTGTTCTCGCCACTT
GTCACCTCCCTCAAACACCTAACAGAGCTCTCTCACAGCACACACATAATCACATGC
GTGCATGCATTATTACACGTGATGCCATGCAAATCTCTTATAGCCTATAAAATTAACT
CATCCGCTTCACTCTTACTCAAACAAACTCATCAATACAAACAAGATTAAAACATA

End -28 untranslated leader TP start
CACGAGGATCCTCAGTCACACAAAGAGTAAAGAAGAACATGGCTTCTCTATGCTCTCT
M A S S M L S

TCCGCTACTATGGTTGCCTCTCCGGCTCAGGCCACTATGGTCGCTCCTTCAACGGACTT
S A T M V A S P A Q A T M V A P F N G L

AAGTCCTCCGCTGCCTCCCAGCCACCCGCAAGGCTAACAAACGACATTACTCCATCACA
K S S A A F P A T R K A N N D I T S I T

FIG.1

2/3

TP End C-4-Oxygenase

AGCAACGGCGGACCGTAACTGCATGTAGAACATGCCATCCGAGTCGTCAGACGCAGCT
 S N G G R V N C M S R M P S E S S D A A

 CGTCCTCGCTAAAGCACGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCACGATG
 R P A L K H A Y K P P A S D A K G I T M

 GCGCTGACCACATCATTGGCACCTGGACCGCAGTGTACACGCAATATTCAAATCAGG
 A L T I I G T W T A V F L H A I F Q I R

 CTACCGACATCCATGGACCAAGCTCACTGGTGCCTGTGTCCGAAGCCACAGCCCAGCTT
 L P T S M D Q L H W L P V S E A T A Q L

 TTGGGCGGAAGCAGCAGCCTACTGCACATCGCTGCAGTCTTCATTGACTTGAGTTCTG
 L G G S S S L L H I A A V F I V L E F L

 TACACTGGTCTATTCATCACCAACACATGACGCAATGCATGGCACCATAGCTTGAGGCAC
 Y T G L F I T T H D A M H G T I A L R H

 AGGCAGCTCAATGATCTCCTTGGAACATCTGCATATCACTGTACGCCCTGGTTGACTAC
 R Q L N D L L G N I C I S L Y A W F D Y

 AGCATGCTGCATCGCAAGCACTGGAGCACCAACCAACTGGCGAAGTGGGGAAAGAC
 S M L H R K H W E H H N H T G E V G K D

 CCTGACTTCCACAAGGAAATCCCGCCTGTCCCCCTGGTCGCCAGCTCATGTCCAGC
 P D F H K G N P G L V P W F A S F M S S

 TACATGTCCCTGTGGCAGTTGCCGGCTGGCATGGTGGCAGTGGTGATGCAAATGCTG
 Y M S L W Q F A R L A W W A V V M Q M L

 GGGCGCCCATGGCAAATCTCTAGTCTCATGGCTGCAGCCCCAATCTTGTCAGCATTC
 G A P M A N L L V F M A A A P I L S A F

 CGCCTCTTCTACTCGGCACCTACCTGCCACACAAGCCTGAGCCAGGCCCTGCAGCAGGC
 R L F Y F G T Y L P H K P E P G P A A G

 TCTCAGGTGATGCCCTGGTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGAGTTTC
 S Q V M A W F R A K T S E A S D V M S F

 CTGACATGCTACCACTTGACCTGCACGGAGCACACAGATGCCCTTGCCCCCTGG
 L T C Y H F D L H W E H H R W P F A P W

 C-4 oxygenase Stop
 TGGCAGCTGCCCACTGCCGCCCTGTCCGGCGTGGCCTGGTGCCTGCCTGGCATGA
 W Q L P H C R R L S G R G L V P A L A *

FIG.1 (cont.)

10/07/04 12

WO 01/20011

PCT/SE00/01767

3 / 3

C-4 oxygenase untranslated region Nos term
CCTGGTCCCTCCGCTGGTGACCCAGCGTCTGCACAAGAGTGTATGGAGCTCGAATTCC
CCGATCGTTCAAACATTGGCAATAAGTTCTTAAGATTGAATCCTGTTGCCGGTCTTG
CGATGATTATCATATAATTCTGTTGAATTACGTTAACGATGTAATAATTAAACATGTAAT
GCATGACGTTATTTATGAGATGGGTTTTATGATTAGAGTCCCGCAATTATAACATTTAAT
ACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCAT
end
CTATGTTACTAGATCGGGAATT

Fig.1 (cont.)

ATTORNEY/DOCKET NO: HOGL3001/REF

DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled: **DNA CONSTRUCT AND ITS USE**

the specification of which (check one):

is attached hereto, or was filed on: 13 September 2000 as PCT International Application Number PCT/SE00/01767

and (if applicable) was amended on:

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56*. I hereby claim foreign priority benefits under *Title 35, United States Code §119* of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)			PRIORITY CLAIMED	
Number	Country	Day/Month/Year Filed	Yes	No
9903336-7	Sweden	17 September 1999	X	

Additional Priority Application(s) Listed on Following Page(s)

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35 U.S. CODE §119(E) OF ANY U.S. PROVISIONAL APPLICATIONS LISTED BELOW.	
Application Number	Day/Month/Year Filed

Additional Provisional Application(s) Listed on Following Page(s)

I hereby claim the benefit under *Title 35, United States Code, §120* of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of *Title 35, United States Code, §112*, I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56* which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Application Number	Filing Date	Status - Patented, Pending or Abandoned

Additional US/PCT Priority Application(s) listed on Following Page(s)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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DATE <u>020304</u>	SIGNATURE <u>Anna-Stina Hoglund</u>

See following page(s) for additional joint inventors.

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(2)

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CONTINUATION OF DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

Page 2

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DATE 020304	SIGNATURE <i>Kjell Stalberg</i>

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DATE	SIGNATURE

FULL NAME OF JOINT INVENTOR	CITIZENSHIP
RESIDENCE ADDRESS	POST OFFICE ADDRESS IS THE SAME AS RESIDENCE ADDRESS UNLESS OTHERWISE SHOWN BELOW
DATE	SIGNATURE

FULL NAME OF JOINT INVENTOR	CITIZENSHIP
RESIDENCE ADDRESS	POST OFFICE ADDRESS IS THE SAME AS RESIDENCE ADDRESS UNLESS OTHERWISE SHOWN BELOW
DATE	SIGNATURE

See following pages for additional joint inventors/priority applications.

(04AUG1998)

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<120> DNA construct and its use

<130> 29295-AstaCarotene

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coding sequence + termination sequence

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tttccaacat tttaaatttc actattggct gaatgcttct tctttgagga agaaacaatt 180

cagatggcag aaatgtatca accaatgcat atatacaaatt gtaccttttgc ttctcaaaac 240

atctatcgga tggttccatt tgctttgtca tccaaatttgact gactactta tattattcac 300

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 120 125 130

cta ctg cac atc gct gca gtc ttc att gta ctt gag ttc ctg tac act 1626
 Leu Leu His Ile Ala Ala Val Phe Ile Val Leu Glu Phe Leu Tyr Thr
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 Gly Leu Phe Ile Thr Thr His Asp Ala Met His Gly Thr Ile Ala Leu
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 Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn Ile Cys Ile Ser Leu
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 His Asn His Thr Gly Glu Val Gly Lys Asp Pro Asp Phe His Lys Gly
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 215 220 225

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 Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp Ala Val Val Met Gln
 230 235 240 245

 atg ctg ggg gcg ccc atg gca aat ctc cta gtc ttc atg gct gca gcc 1962
 Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val Phe Met Ala Ala Ala
 250 255 260

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 Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe Gly Thr Tyr Leu Pro
 265 270 275

 cac aag cct gag cca ggc cct gca gca ggc tct cag gtg atg gcc tgg 2058
 His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser Gln Val Met Ala Trp
 280 285 290

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 Cys Tyr His Phe Asp Leu His Trp Glu His His Arg Trp Pro Phe Ala
 310 315 320 325

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 Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu Ser Gly Arg Gly Leu
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 Val Pro Ala Leu Ala
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<223> Description of Artificial Sequence: deduced fusion protein of
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20 25 30Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
35 40 45Asn Gly Gly Arg Val Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser
50 55 60Asp Ala Ala Arg Pro Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser
65 70 75 80Asp Ala Lys Gly Ile Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr
85 90 95Ala Val Phe Leu His Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met
100 105 110Asp Gln Leu His Trp Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu
115 120 125Gly Gly Ser Ser Ser Leu Leu His Ile Ala Ala Val Phe Ile Val Leu
130 135 140Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His Asp Ala Met His
145 150 155 160Gly Thr Ile Ala Leu Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn
165 170 175Ile Cys Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg
180 185 190Lys His Trp Glu His His Asn His Thr Gly Glu Val Gly Lys Asp Pro
195 200 205Asp Phe His Lys Gly Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe
210 215 220Met Ser Ser Tyr Met Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp
225 230 235 240Ala Val Val Met Gln Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val
245 250 255

Phe Met Ala Ala Ala Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe
260 265 270

Gly Thr Tyr Leu Pro His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser
275 280 285

Gln Val Met Ala Trp Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val
290 295 300

Met Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His His
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ctc tct tcc gct act atg gtt gcc tct ccg gct cag gcc act atg gtc Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala Gln Ala Thr Met Val 10 15 20	1242
gct cct ttc aac gga ctt aag tcc tcc gct gcc ttc cca gcc acc cgc Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala Phe Pro Ala Thr Arg 25 30 35	1290
aag gct aac aac gac att act tcc atc aca agc aac ggc gga cgc gtt Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser Asn Gly Gly Arg Val 40 45 50	1338
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gcg cta aag cac gcc tac aaa cct cca gca tct gac gcc aag ggc atc Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser Asp Ala Lys Gly Ile 70 75 80 85	1434
acg atg gcg ctg acc atc att ggc acc tgg acc gca gtg ttt tta cac Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr Ala Val Phe Leu His 90 95 100	1482
gca ata ttt caa atc agg cta ccg aca tcc atg gac cag ctt cac tgg Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met Asp Gln Leu His Trp 105 110 115	1530
ttg cct gtg tcc gaa gcc aca gcc cag ctt ttg ggc gga agc agc agc Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu Gly Gly Ser Ser Ser 120 125 130	1578
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ggc cta ttc atc acc aca cat gac gca atg cat ggc acc ata gct ttg	1674
Gly Leu Phe Ile Thr Thr His Asp Ala Met His Gly Thr Ile Ala Leu	
150 155 160 165	
agg cac agg cag ctc aat gat ctc ctt ggc aac atc tgc ata tca ctg	1722
Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn Ile Cys Ile Ser Leu	
170 175 180	
tac gcc tgg ttt gac tac agc atg ctg cat cgc aag cac tgg gag cac	1770
Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg Lys His Trp Glu His	
185 190 195	
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His Asn His Thr Gly Glu Val Gly Lys Asp Pro Asp Phe His Lys Gly	
200 205 210	
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Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe Met Ser Ser Tyr Met	
215 220 225	
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Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp Ala Val Val Met Gln	
230 235 240 245	
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Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val Phe Met Ala Ala Ala	
250 255 260	
cca atc ttg tca gca ttc cgc ctc ttc tac ttc ggc act tac ctg cca	2010
Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe Gly Thr Tyr Leu Pro	
265 270 275	
cac aag cct gag cca ggc cct gca gca ggc tct cag gtg atg gcc tgg	2058
His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser Gln Val Met Ala Trp	
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Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val Met Ser Phe Leu Thr	
295 300 305	
tgc tac cac ttt gac ctg cac tgg gag cac cac aga tgg ccc ttt gcc	2154
Cys Tyr His Phe Asp Leu His Trp Glu His His Arg Trp Pro Phe Ala	
310 315 320 325	
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Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu Ser Gly Arg Gly Leu	
330 335 340	
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Val Pro Ala Leu Ala	
345	
agtgtcatgg agctcgaatt tccccgatcg ttcaaacatt tggcaataaa gtttcttaag	2317
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agtcccgcaa ttatacattt aatacgcgat agaaaacaaa atatagcgcg caaacttagga	2497
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<210> 2

<211> 346

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: deduced fusion protein of
transit peptide + peptide with beta-carotene C-4 oxygenase activity

<400> 2

Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala

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Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
20 25 30Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
35 40 45Asn Gly Gly Arg Val Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser
50 55 60Asp Ala Ala Arg Pro Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser
65 70 75 80Asp Ala Lys Gly Ile Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr
85 90 95Ala Val Phe Leu His Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met
100 105 110Asp Gln Leu His Trp Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu
115 120 125Gly Gly Ser Ser Ser Leu Leu His Ile Ala Ala Val Phe Ile Val Leu
130 135 140Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His Asp Ala Met His
145 150 155 160Gly Thr Ile Ala Leu Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn
165 170 175Ile Cys Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg
180 185 190Lys His Trp Glu His His Asn His Thr Gly Glu Val Gly Lys Asp Pro
195 200 205Asp Phe His Lys Gly Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe
210 215 220Met Ser Ser Tyr Met Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp
225 230 235 240Ala Val Val Met Gln Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val
245 250 255

Phe Met Ala Ala Ala Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe
260 265 270

Gly Thr Tyr Leu Pro His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser
275 280 285

Gln Val Met Ala Trp Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val
290 295 300

Met Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His His
305 310 315 320

Arg Trp Pro Phe Ala Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu
325 330 335

Ser Gly Arg Gly Leu Val Pro Ala Leu Ala
340 345